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Phorbol esters inhibit low density lipoprotein processing by cultured human fibroblasts

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A 24 h pretreatment of MRC5 fibroblasts with the protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induced a marked decrease in low density lipoprotein (LDL) internalization and degradation; the maximal effect (about 55% decrease) was observed for 10^{-7} M TPA. LDL binding was reduced about 35–40%. A significant decrease (about 25%) in LDL internalization was observed after a 2 h incubation of cells with the drug, but longer incubation times (4–6 h) led to a greater effect. Another tumor promoter, phorbol 12,13-dibutyrate decreased LDL internalization by about 35%, whereas the non-tumor promoting 4α-phorbol 12,13-didecanoate had no effect. The protein kinase C inhibitor α-cobrotoxin partially antagonized the inhibitory effect of TPA on LDL internalization. The non-phorbol tumor promoter mezerein, another protein kinase C activator, decreased LDL uptake by about 50%. Finally, it was found that TPA had no significant effect on the affinity of the receptor for the LDL. These results suggest a role for protein kinase C in the LDL pathway in cultured human fibroblasts.

LDL *Phorbol ester* *Protein kinase C* *(Human fibroblast)*

1. INTRODUCTION

LDL catabolism is mainly achieved by a receptor-mediated endocytic pathway, which results in down-regulation of sterol metabolism [1,2]. In previous studies, we demonstrated a significant decrease in LDL internalization in cultured human fibroblasts pretreated with the calcium ionophore A23187, which suggested a role for Ca^{2+} in the control of LDL endocytosis [3]. Several reports pointed out the synergy between Ca^{2+} and protein kinase C activation in various cellular processes such as steroidogenesis [4], histamine release from mast cells [5] or insulin secretion [6]. Tumor-promoting phorbol esters, which activate protein kinase C [7,8], have been demonstrated to inhibit epidermal growth factor [9,10] or insulin binding [11]. More recently, Rouis et al. [12] reported that phorbol esters inhibit LDL processing in the monocyte-like cell strain U 937. In contrast, Shoyab et al. [13] found no effect of phorbol esters on LDL binding by cultured human fibroblasts. In this study, we re-examined the effects of various phorbol esters on LDL processing by cultured human fibroblasts, and found that tumor-promoting phorbol esters significantly reduced LDL binding and markedly inhibited LDL internalization. Non-tumor-promoting phorbol esters had no effect.

Abbreviations: TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; PDBu, phorbol 12,13-dibutyrate; α-PDD, 4α-phorbol 12,13-didecanoate; LDL, low density lipoprotein

2. MATERIALS AND METHODS

2.1. *Reagents*

TPA, PDBu, α-PDD, mezerein and α-cobrotoxin were from Sigma.

2.2. *Cell culture*

MRC5 (human foetal lung fibroblasts) were purchased from Biomérieux and kept in Ham F10

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medium supplemented with 10% foetal calf serum (Gibco) in 75 cm^2 Falcon flasks at 37°C, in a 5% CO_2 humidified atmosphere. For experiments, cells were grown in 30 mm petri dishes (Nunc) in Ham F10 medium supplemented with 2% Ultroser G (Industries Biologiques Françaises) and effectors as specified below.

2.3. *LDL preparation and labelling*

LDL was prepared from normal human serum according to Havel et al. [14] and labelled as described in [15], using $Na^{125}I$ (Amersham, 13–17 Ci/mg). The specific activity of the LDL was about 200–300 dpm/ng protein. Protein determination was done according to Lowry et al. [16].

2.4. *Effect of phorbol esters, mezerein and α-cobrotoxin on LDL binding, internalization and degradation*

Cells were incubated for 2–24 h in Ham F10 medium + 2% Ultroser G in the absence or presence of different protein kinase C effectors (phorbol esters, mezerein, α-cobrotoxin) at the indicated concentrations. After preincubation, the cells were washed 3 times with a phosphate-buffered solution, and then LDL binding, internalization and degradation were studied according to Goldstein and Brown [1]. Incubations were performed for 2 h at 4°C for binding studies, and 4 h at 37°C for uptake and degradation studies, in 0.5 ml Ham F10, 10 mM Hepes (pH 7.4) medium. The final concentration of LDL in the incubation medium was 10 μg/ml. After incubation, cells were washed 4 times with a phosphate-buffered solution (pH 7.4), harvested with a rubber policeman and centrifuged for 5 min at 400 × *g*. The radioactivity associated with the pellet was measured with a Packard 256 gamma counter. For the study of ^{125}I-LDL degradation, the incubation medium (0.5 ml) was removed before cell washing and 0.25 ml of 1% bovine serum albumin + 0.25 ml of 50% trichloroacetic acid were added. The precipitate was centrifuged for 10 min at 4000 × *g*. 0.01 ml of 40% KI and 0.05 ml of 30% H_2O_2 were then added to 0.5 ml of the supernatant. After mixing, the samples were maintained for 30 min at 4°C, then extracted with 2 ml chloroform and the radioactivity counted on 0.2 ml of the upper phase.

In experiments designed to determine whether the receptor number or the affinity is reduced, cells were incubated with increasing concentrations of ^{125}I-LDL (1.25–50 μg/ml) in the absence or presence of 500 μg/ml unlabelled LDL. After a 2 h incubation at 4°C, the radioactivity associated with the cells was measured as described above.

3. RESULTS AND DISCUSSION

Fig.1 displays the effect of the protein kinase C activator TPA on LDL binding, uptake and degradation in relation to the drug concentration. A dose-dependent decrease was observed, with a maximal reduction of about 35–40% of LDL binding, and 50–60% decrease in LDL uptake and degradation for 10^{-7} M TPA.

In the following experiments, we studied especially the effect of protein kinase C effectors on LDL internalization and degradation.

It can be seen in fig.2 that a small effect was obtained for an incubation time as short as 2 h (about

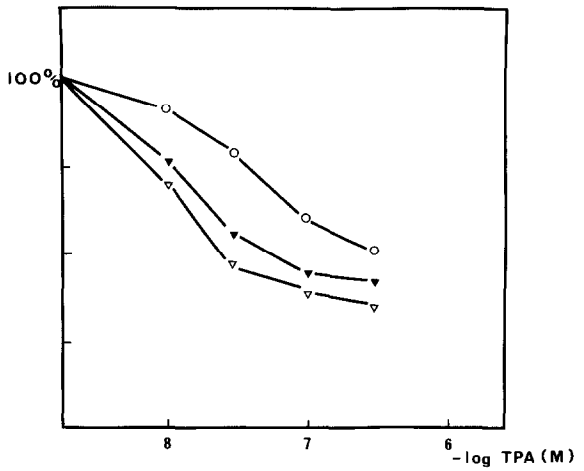

Fig.1. Effect of TPA on LDL binding (○), internalization (▽) and degradation (▼) by MRC5 human fibroblasts, in relation to drug concentration. Cells were preincubated 24 h with TPA in Ham F10 medium supplemented with 2% Ultroser G, washed 3 times and then LDL binding, internalization and degradation were determined as described in section 2 with ^{125}I-LDL (10 μg/ml). Results are expressed in % of controls (100%: 134 ± 22 ng LDL bound, 570 ± 96 ng LDL internalized and 1368 ± 232 LDL degraded per mg cell protein); mean of 3 experimental values.

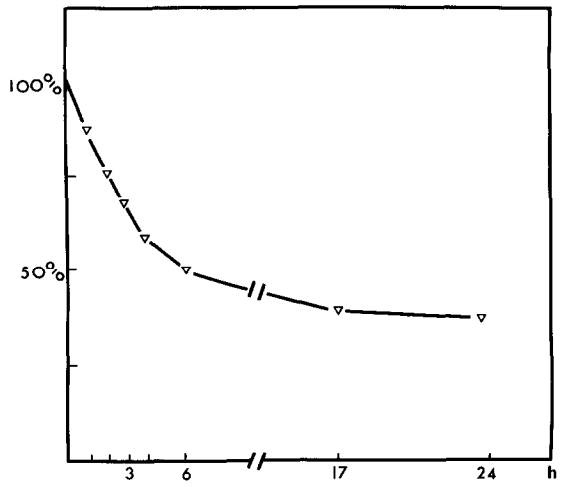

Fig.2. Effect of 5×10^{-7} M TPA on LDL internalization by MRC5 fibroblasts in relation to the preincubation time of cells with the drug. Preincubation was performed in Ham F10 medium supplemented with 2% Ultroser G. Cells were then washed and LDL internalization was measured as described in section 2 with ^{125}I-LDL (10 μg/ml). Results are expressed in % of controls (100%: 579 ± 96 ng LDL internalized); mean of 3 or 4 experimental values.

25% decrease), but a longer incubation time led to a greater reduction in LDL uptake. 80% of the maximal effect was obtained within 6 h of preincubation in the presence of 10^{-7} M TPA.

To study the relationship between the tumor-promoting activity of drugs and their effect on LDL processing by MRC5 fibroblasts, we compared various tumor and non-tumor promoters for their capacity to reduce LDL uptake and degradation: TPA and PDBu, which are tumor-promoting phorbol esters shown to activate protein kinase C; α-PDD, which has no tumor-promoting effect, and does not activate protein kinase C; and mezerein, a non-phorbol tumor promoter which has recently been reported to activate strongly protein kinase [17]. The effect of cobrotoxin, a potent inhibitor of protein kinase C [18], has also been investigated. The results of these studies are summarized in table 1.

It can be observed that the tumor-promoting protein kinase C activator PDBu was almost as effective as TPA in reducing LDL processing by MRC5 fibroblasts (about 40% decrease was obtained with PDBu 10^{-7} M). In contrast, α-PDD had no significant effect on LDL uptake and degradation. The non-phorbol tumor promoter and protein kinase C activator mezerein reduced LDL uptake and degradation to about 50% of controls at 10^{-6} M. Finally, the protein kinase C inhibitor cobrotoxin alone slightly stimulated LDL internalization and degradation, and antagonized the inhibitory effect of TPA when cells were pretreated simultaneously with these drugs.

Experiments were then performed to determine

Table 1

Effect of various phorbol esters (TPA, PDBu, α-PDD) and non-phorbol protein kinase C effectors (mezerein, activator; cobrotoxin, inhibitor) on LDL internalization and degradation by MRC5 human fibroblasts

Addition	Internalization (ng/mg protein)	Degradation (ng/mg protein)
None	570 ± 96 (100%)	1368 ± 232 (100%)
TPA (10^{-7} M)	268 ± 38 (47%)	616 ± 92 (45%)
PDBu (10^{-7} M)	331 ± 50 (58%)	862 ± 134 (63%)
α-PDD (10^{-7} M)	558 ± 78 (98%)	1310 ± 225 (96%)
Cobrotoxin (50 μg/ml)	706 ± 122 (124%)	1532 ± 245 (112%)
TPA (10^{-7} M) + cobrotoxin (50 μg/ml)	421 ± 65 (74%)	985 ± 167 (72%)
Mezerein (10^{-6} M)	296 ± 46 (52%)	656 ± 92 (48%)

Cells were preincubated for 24 h with the drugs at the indicated concentrations in Ham F10 medium supplemented with 2% Ultroser G, and then were washed 3 times before LDL internalization and degradation were measured with ^{125}I-LDL (10 μg/ml). Results are mean of 3 experimental values ± SD

if the number of receptors was reduced by TPA or if the affinity of the receptor for the LDL was affected. Linearization plot of the data according to Riggs [19] showed (fig.3) that the K_m (point of intersection with the y axis = $-K_m$) was not significantly modified under 5×10^{-7} M TPA (about 6 μM with or without TPA). In contrast, the V_{max} was about 40–45% reduced in treated cells as compared to untreated cells (7.5 ng LDL bound/μg of cell protein in the presence of TPA, and 13 ng/μg in controls). Thus, the number of LDL receptors at the cell surface appears to be decreased without any significant modification of their affinity for the lipoprotein.

Our results demonstrate that the tumor-promoting phorbol esters TPA and PDBu significantly inhibit LDL processing by MRC5 fibroblasts. This effect appears to be probably related to the activating effect of these drugs on protein kinase C activity, as the non-phorbol protein kinase C activator mezerein was also effective. These results are not in accordance with those of Shoyab et al. [13], who found no effect of TPA on LDL binding by foreskin fibroblasts. It must be considered that the effect of TPA may be dependent on the cell strain, although we generally observed similar regulation of LDL and sterol metabolism in skin and MRC5 fibroblasts. It must be also emphasized that the experimental conditions were notably different; in our experiments cells were incubated with TPA in Ham F10 medium devoid of lipoproteins, whereas in Shoyab's experiments it seems that cells were grown in Dulbecco's medium supplemented with 10% foetal calf serum. In the latter experimental conditions, fibroblasts exhibit a very low level of LDL receptors (about 15–20% as compared to the number of receptors of cells maintained in a medium devoid of lipoproteins). In this case, it may be difficult to point out a reduction in LDL uptake by TPA.

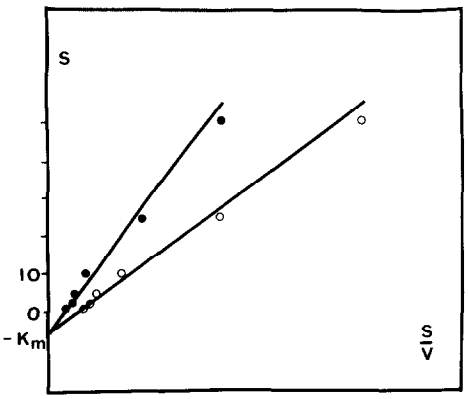

Fig.3. Effect of 5×10^{-5} M TPA on the affinity of receptors for LDL. After a 24 h preincubation with 5×10^{-5} M TPA, cells were washed, then incubated for 2 h at 4°C with increasing ^{125}I-LDL concentrations (1.25–50 μg/ml) in the absence or presence of 500 μg/ml unlabelled LDL. (●) Controls, (○) 5×10^{-7} M TPA. Linearization plot of the data was done according to Riggs [19]. Each point is the mean of 3 experimental values. Intersection with y axis = $-K_m$; slope = V_{max}.

Our findings may be compared to those of Rouis et al. [12] who recently pointed out a decrease in LDL binding by TPA in a different system; the monocyte-like strain U 937. However, there are marked differences in the kinetics of TPA effect in fibroblasts and in the monocyte-like strain U 937. In the U 937 strain, the maximal effect of TPA was obtained for very short preincubation times of cells with the drug (less than 1 h), whereas we only observed a half-maximal effect (about 25% reduction) for a 2 h preincubation of fibroblasts with TPA. A 4–6 h incubation time is necessary to obtain the maximal effect (55% reduction) in fibroblasts.

The mechanism whereby tumor-promoting phorbol esters inhibit LDL uptake by fibroblasts is still unknown. The fact that mezerein, another protein kinase C activator, mimics the effect of TPA, suggests that protein kinase C is involved in the phenomenon. This hypothesis is further supported by the ability of cobrotoxin, a protein kinase C inhibitor [18], to antagonize the TPA-induced reduction of LDL uptake. Rouis et al. [12] suggested that TPA could induce phosphorylation of the receptor and the formation of a cryptic pool, by inhibition of receptor recycling. In fact, LDL receptor recycling is very rapid: about 9 min per cycle as described by Anderson et al. [20]. Thus, the inhibition of the LDL receptor recycling is compatible with the rapid effect of TPA described by Rouis et al. [12] in the U 937 strain, since these authors found significant inhibition of LDL binding for a preincubation time as short as 15 min. In our case, the absence of effect of TPA under 1 h suggests that other mechanisms may be involved in fibroblasts.

In conclusion, our findings point out an inhibitory effect of phorbol and non-phorbol protein kinase C activators on LDL processing by human fibroblasts. This suggests a role for protein kinase C in the control of receptor-mediated LDL endocytosis in these cells.

Experiments are now being performed to elucidate the actual mechanism of the inhibition of LDL uptake by phorbol esters and mezerein in human fibroblasts, especially the determination of the state of phosphorylation of the LDL receptor under TPA treatment.

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